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Identification of Members of the Metabolically Active Microbial Populations Associated with Beggiatoa Species Mat Communities from Gulf of Mexico Cold-Seep Sediments In this study, the composition of the metabolically active fraction of the microbial community occurring in Gulf of Mexico marine sediments (water depth, 550 to 575 m) with overlying filamentous bacterial mats was determined. The mats were mainly composed of either orange- or white-pigmented Beggiatoa spp. Complementary 16S ribosomal DNA (crDNA) was obtained from rRNA extracted from three different sediment depths (0 to 2, 6 to 8, and 10 to 12 cm) that had been subjected to reverse transcription-PCR amplification. Domain- specific 16S PCR primers were used to construct 12 different 16S crDNA libraries containing 333 Archaea and 329 Bacteria clones. Analysis of the Archaea clones indicated that all sediment depths associated with overlying orange- and white-pigmented microbial mats were almost exclusively dominated by ANME-2 (95% of total Archaea clones), a lineage related to the methanogenic order Methanosarcinales. In contrast, bacterial diversity was	Xác định các thành viên của quần thể vi sinh vật có hoạt tính trao đổi chất gắn với quần xã thảm của các loài Beggiatoa từ trầm tích phun lạnh ở Vịnh Mexico checked 28/7 Trong nghiên cứu này, chúng tôi nghiên cứu hoạt động trao đổi chất của quần thể vi sinh vật xảy ra trong trầm tích biển vịnh Mexico (độ sâu của nước, 550-575 m) được bao phủ bởi các thảm vi khuẩn dạng sợi. Các thảm này chủ yếu bao gồm Beggiatoa spp. sắc tố cam và trắng. DNA ribosome 16S bổ sung (crDNA) thu được từ rRNA chiết được từ 3 độ sâu trầm tích khác nhau (0 đến 2, 6 đến 8, 10 đến 12 cm) trải qua quá trình khuếch đại PCR phiên mã ngược. Các Primer (mồi) PCR 16S miền chuyên biệt được sử dụng để xây dựng 12 thư viện 16S crDNA khác nhau chứa 333 dòng vô tính cổ khuẩn và 329 dòng vô tính vi khuẩn. Phân tích các dòng vô tính cổ khuẩn cho thấy, ANME-2 (95% trong tổng số các dòng vô tính cổ khuẩn), một dòng liên quan đến bộ Methanosarcinales sinh methan hầu như chiếm ưu thế ở tất cả các độ sâu trầm tích ứng với các thảm sắc tố cam và trắng. Ngược lại, các dòng vô tính gắn với vi khuẩn lại có sự đa dạng	

considerably higher, with the dominant phylotype varying by sediment depth. An equivalent number of clones detected at 0 to 2 cm, representing a total of 93%, were related to the y and 8 classes of Proteobacteria, whereas clones related to 8-Proteobacteria dominated the metabolically active fraction of the bacterial community occurring at 6 to 8 cm (79%) and 10 to 12 cm (85%). This is the first phylogenetics-based evaluation of the presumptive metabolically active fraction of the Bacteria and Archaea community structure investigated along a sediment depth profile in the northern Gulf of Mexico, a hydrocarbon-rich cold-seep region.	hơn đáng kể, thể hiện ở chỗ mỗi độ sâu trầm tích lại có một vi khuẩn chuyên biệt chiếm ưu thế. Số dòng tương đương phát hiện được ở độ sâu từ 0 đến 2 cm, đại diện cho tổng cộng 93%, có họ hàng với các lớp…. và …. của Proteobacteria, trong khi đó, các dòng vô tính có họ hàng với..-Proteobacteria chiếm ưu thế trong hoạt động trao đổi chất của quần thể vi khuẩn xuất hiện ở độ sâu từ 6 đến 8 cm (79%) và từ 10 đến 12 cm (85%). Đây là đánh giá dựa trên phả hệ đầu tiên về khía cạnh trao đổi chất của cấu trúc quần thể cổ khuẩn và vi khuẩn được nghiên cứu theo biên dạng độ sâu trầm tích ở phía bắc vịnh Mexico, một khu vực phun lạnh giàu hydrocarbon.	
The Gulf of Mexico is a dynamic environment containing active venting and seepage of hydrocarbons. The upward thrust of salt diapirs forms faults that act as conduits from subsurface oil and gas reservoirs through the sediment layers (42, 58). Faults reaching the surface can facilitate the formation of surface-breaching gas hydrate mounds (reviewed in reference 4) and actively venting hydrocarbon plumes (7, 11, 39, 40). In contrast to hydrothermal seeps, these features are collectively known as cold seeps due to low-level geological	hoạt động mạnh với các hoạt động thoát và rò rỉ hydrocacbon Sức đóng vai trò như các các mỏ dầu khí dưới bề mặt nổi lên bề mặt Trái ngược với hiện tượng phun thủy nhiệt, những	

heating. owing to the extensive oil and gas reserves, a primary focus of long-term research in the Gulf of Mexico has been the characterization of the physical geology of the system (30, 39). Surprisingly, Gulf of Mexico cold-seep chemosynthetic-based ecosystems were not reported until 1989 (27), and the ecosystem's primary energy source (CH4) was not linked to gas hydrate decomposition until 1994 (5).	đặc trưng này được gọi chung là phun lạnh do nó xảy ra ở nhiệt độ địa chất thấp nguồn dự trữ dầu khí dồi dào là xác định đặc điểm địa chất cơ sở các hệ sinh thái dựa trên hóa tổng hợp phun lạnh ở Vịnh Mexico chưa được nghiên cứu người ta chưa thấy được mối liên hệ giữa nguồn năng lượng cơ bản của hệ sinh thái (CH4) với sự phân hủy khí hydrat cho đến năm 1994 (5).	
A more thorough characterization of an ecosystem, how-ever, requires identification of the mechanisms and biota responsible for energy transfer and the cycling of nutrients. Owing to the water depth at Gulf of Mexico cold seeps, che-mosynthesis rather than photosynthesis predominates (41). As has been shown for hydrothermal seep ecosystems, energy transfer from chemosynthetic microorganisms to higher trophic levels is mediated by primary consumers, including symbiont-containing macrofauna and free-living heterotrophic microorganisms (9, 10, 12, 33).		

Whereas numerous reports characterizing tubeworm and mussel symbiotic associations with chemoautotrophic microbes have been published (6, 10, 23), that portion of the chemosynthesis-based community in the Gulf of Mexico comprised of nonsymbiotic and free-living microbes has been much less studied (24, 31, 59). For example, vast aggregations of dense microbial mats on the sediment surface are readily visible to the naked eye. These mats are mainly composed of large (cell diameter, 12 to 160 ^m) (13, 29), pigmented (orange and white) and unpigmented vacuolate sulfur bacteria, Beggiatoa spp. (2, 33, 57).

Such mat- and sediment-associated microbial communities have been shown to support high rates of sulfate reduction (3, 22, 36, 57) and oxidation (25, 36, 49), nitrate reduction (36, 43, 49), and anaerobic methane oxidation (3, 22). Interestingly, as potentially critical as these microbial communities are to Gulf of Mexico cold-seep ecosystem productivity, no detailed information describing the composition of the metabolically

active microbes and their spatial and/or temporal structures is available. In the present study, depth profiles of sediments with two different overlying types of microbial mats, composed mainly of orange- and white-pigmented Beggiatoa spp., were collected from a manned submersible at two different cold-seep locations (550 to 575 m water depth). The primary objective in this study was to characterize the metabolically active fraction of the sediment microbial communities associated with the microbial mats. Total rRNA was extracted from three sediment depths (0 to 2, 6 to 8, and 10 to 12 cm) and subjected to reverse transcription-PCR with primers specific for the Bacteria and Archaea. This is among the first phylogenetic surveys to be conducted on Gulf of Mexico seep sediment microbial communities directly associated with overlying microbial mats and the first survey describing the metabolically active fraction of the microbial communities in Gulf of Mexico sedimentary habitats.	[redacted]	

MATERIALS AND METHODS

Gulf of Mexico site description and sample collection. The study sites are located in the northern Gulf of Mexico continental slope province. The sites, GC185 (Bush Hill; 550 m depth) and GC234 (575 m depth), are located at 27°46'N, 91°30'W, and 27°44'N, 91°13'W, respectively. Both of these locales contained visible oil and gas seepage, surface-breaching gas hydrate, and exten-sive (several meters in diameter) microbial mats. Sediment cores from both sites were obtained from areas containing mainly either orange- or white-pigmented Beggiatoa sp. mats with the manned submersible Johnson Sea Link during July 2002. Sediment cores (7.2 cm inner diameter, 15 to 20 cm average length) were immediately sectioned at 2-cm intervals and stored in liquid N2 until further processing. Direct cell counts were performed on aliquots (0.5 g wet weight) as previously described (38).

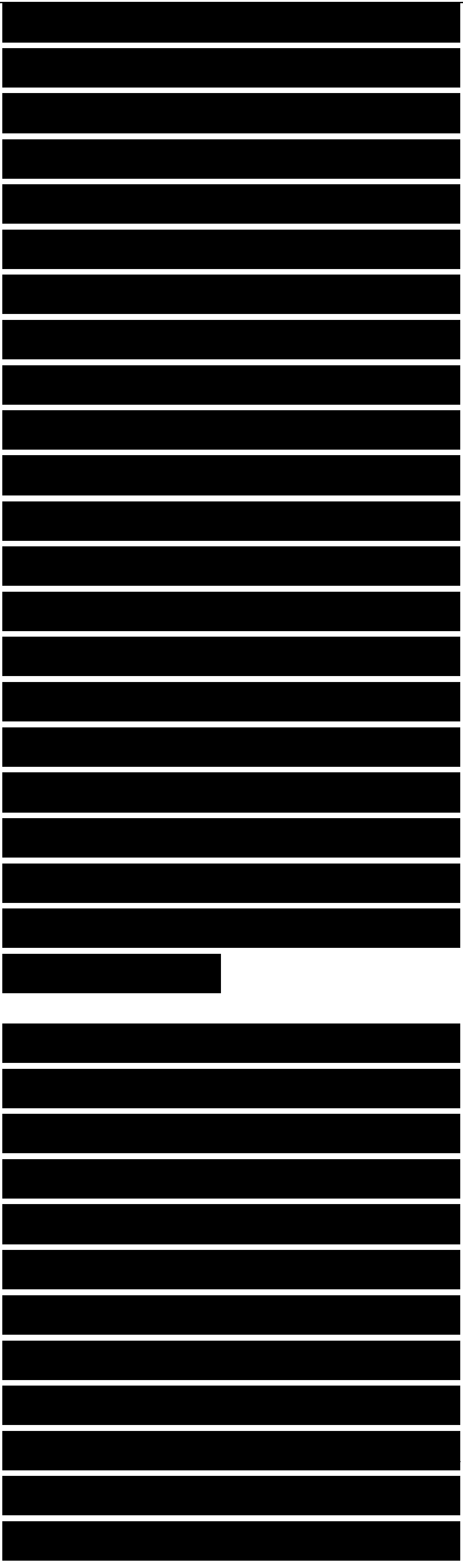
Preparation of reagents and materials used for RNA extractions. Prior to nucleic acid extraction, RNases were

removed from solutions and solids by treating stock solutions and water with 0.1% diethylpyrocarbonate overnight at 37° C and autoclaving. All glassware and nonplastics were baked at 250° C for 24 h. All surfaces and plastics were cleaned with RNase Erase (iCN Biomedicals inc., Aurora, ohio) to remove contaminating RNases during shipboard and laboratory manipulations. RNA isolation. Total ribonucleic acids were extracted as described by Hurt et al. (20) from 10 g (wet weight) of sediment sampled in triplicate from each sediment depth (0 to 2, 6to 8, and 10to 12 cm). Inbrief, sediment samples stored in liquid N2 were repeatedly thawed by physical grinding in the presence of a denaturing solution (4 M guanidine isothiocyanate, 10 mM Tris-HCl [pH 7.0], 1 mM EDTA, 0.5% 2-mercaptoethanol) and refrozen by immersion in liquid N2. The sediment samples were incubated for 30 min at 65°C in pH 7.0 extraction buffer (100 mM sodium phosphate [pH 7.0], 100 mM Tris-HCl [pH 7.0], 100		

mM EDTA [pH 8.0], 1.5 M NaCl, 1% hexadecyltrimethylammonium bromide [CTAB], and 2% sodium dodecyl sulfate [SDS]) and centrifuged (1,800 X g for 10 min).		
The supernatants from three separate extractions were pooled, extracted with 24:1 (vol/vol) chloroform-isoamyl alcohol, and centrifuged (1,800 X g for 20 min). The nucleic acids were precipitated at room temperature with isopropanol (30 min), pelleted by centrifugation (16,000 X g for 20 min), resuspended in diethylpyrocarbonate-treated water, and subsequently purified into RNA-only aliquots (20).		
Reverse transcription and amplification of rRNA. Aliquots of rRNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Invitrogen). RNA was initially denatured by heating (65°C) for 10 min. The reverse transcription reaction mix consisted of 5 fjuM of a 16S rRNA reverse primer amplifying either domain-		

specific Bacteria, DXR518 (5'-CGTATTACCGCGGCTGCTG G-3') (34), or Archaea, Ar958r (5'-YCCgGcGTTGAMTCCAATTT -3') (8), 50 to 100 ng of denatured RNA, and 200 fjuM deoxynucleoside triphosphate mix. The mixture was incubated for 5 min at 65°C and 2 min at 4°C, followed by addition of 1 X first-strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl2) and 75 U of RNase inhibitor and heating at 37°C for 2 min. A 200-U aliquot of Moloney murine leukemia virus reverse transcriptase was added prior to a 50-min incu-bation at 37°C that resulted in transcription of the RNA into complementary 16S ribosomal DNA (crDNA). The crDNA end product was used as the template for a standard PCR. Possible DNA contamination of RNA templates was routinely monitored by PCR amplification of aliquots of RNA that were not reverse transcribed. No contaminating DNA was detected in any of these reactions.		

The primers used for standard PCR amplification included the above reverse primers (DXR518 and Ar958r) and 16S rDNA forward domain-specific Bacteria, 27F (5'-AGAGTTT GATCƠTGGCTCAG-3'), and Archaea, A341f(5'-CCTAIGGGGIGCAICAG- 3') (56), primers. The PCR mix contained 10 to 50 ng of crDNA, 1X PCR buffer (Stratagene), 1.5 mM MgƠ2, 200 fjuM each deoxynucleoside triphosphate, 1 pmol of each forward and reverse primer, and 0.025 U of TaKaRa Taq fjul-1. Ampli- cons were analyzed on 1.0% agarose gels run in Tris-borate-EDTA buffer stained with ethidium bromide and UV illuminated.



Environmental clone library construction. Purified pooled amplicons repre-senting 16S crDNA sequences were cloned into the ToPo TA cloning vector pCR2.1 according to the manufacturer’s instructions (Invitrogen). Clones de-noted in Tables 1 and 2 as WB and B were obtained from sediments with overlying white- or orange-pigmented mats, respectively. In addition, the desig-nations 47 and 52 denote Gulf of Mexico

sites GC185 and GC234, respectively, and 02, 68, and 10 denote depths of 0 to 2, 6 to 8, and 10 to 12 cm, respectively.		
Inserts were subsequently PCR amplified from lysed colonies with primers specific for either the vector, M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGaC-3'), or the Archaea amplicons, A341f (56) and Ar958r (8).		
Vector-specific M13F/R primers were used to amplify inserts from bacterial clones obtained with the 27F and DXR518 primers to prevent amplification of the Escherichia coli host 16S rDNA gene.		
PCR products were digested (2 h, 37°C) with MspI and HhaI for bacterial clones and with HhaI and RsaI for archaeal clones.		
Clones were grouped according to restriction fragment length polymorphism (RFLP) banding		

patterns, and representative clones were sequenced as previ-ously described (31). RFLP groups containing two or more members had rep-resentative clones sequenced.

Multiple representative clones were sequenced from RFLP groups containing five or more members to verify group integrity. A limited number of clones from those RFLP groups containing a single member were also sequenced.

All calculations were based upon the number of clones incorporated in RFLP groups that had representative clones sequenced. Se-quencing was performed at the Georgia Institute of Technology core DNA facility with a BigDye Terminator v3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Gene Analyzer; Applied Biosystems). Inserts were sequenced multiple times on each strand. Prior to comparative sequence analysis, vector sequences flanking the bacterial 16S crDNA insert were manually removed.

Phylogenetic and rarefaction

analysis. Sequence analysis was preformed as previously described (31). Multiple sequences of individual inserts were initially aligned with the program BLAST 2 Sequences (50) available through the National Center for Biotechnology Information and assembled with the program BioEdit v5.0.9 (16). Sequences were checked for chimeras with Chimera Check from Ribosomal Database Project II (28). Sequences from this study and refer-ence sequences, as determined by BLAST analysis, were subsequently aligned with CLUSTALX v1.81 (52).		
An average of 500 (Bacteria clones) to 600 (Archaea clones) nucleotides were included in the final phylogenetic analyses. Neighbor-joining trees were created from the shortened sequence alignments. The bootstrap data represent 1,000 samplings. The final trees were viewed with NJPlot (37) and TreeView v1.6.6, available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html. Rarefaction analysis was performed with the equations as described in Heck et al. (18).	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html	

Standard calculations were used to produce the curve with the total number of clones obtained compared to the number of clones representing each unique RFLP pattern. The percent coverage (C) of the clone libraries was calculated according to the equation $C = [1 - (nj/N)] \times 100$ (15, 32), where nj is the number of unique clones as determined by RFLP analysis and N is the total number of clones in the library.

Nucleotide sequence accession numbers. The 61 16S crDNA gene nucleotide sequences have been deposited in the GenBank database under accession numbers AY32449 to AY324550.

RESULTS

RNA was extracted from three different sediment depths (0 to 2, 6 to 8, and 10 to 12 cm) from sites with overlying orange- or white-pigmented microbial mats from gas hydrate- bearing cold-seep locations in the Gulf of Mexico. The purified RNA was of sufficient quality and quantity to be reverse transcribed, amplified with 16S domain-specific PCR primers, and cloned.

Microbial cell numbers in sediments with overlying orange- and white-pigmented mats were quantified by direct microscopy. Cell counts per gram of sediment were 1.1 X 108 (0 to 2 cm), 4.6 X 107 (6 to 8 cm), and 4.4 X 107 (10 to 12 cm) for the orange-pigmented microbial mat samples and 1.9 X 107 (0 to 2 cm), 7.3 X 106 (6 to 8 cm), and 1.7 X 107 (10 to 12 cm) for the white-pigmented microbial mat samples.

Direct micro-scopic examination of individual giant filaments from orange- and white-pigmented mats revealed few if any gross morphological differences (data not shown). These observations were consistent with previous assignments of these filamentous bacteria to the genus Beggiatoa (33). It should be noted, however, that while Beggiatoa spp. dominated these mats, other as yet unidentified microorganisms were also present.

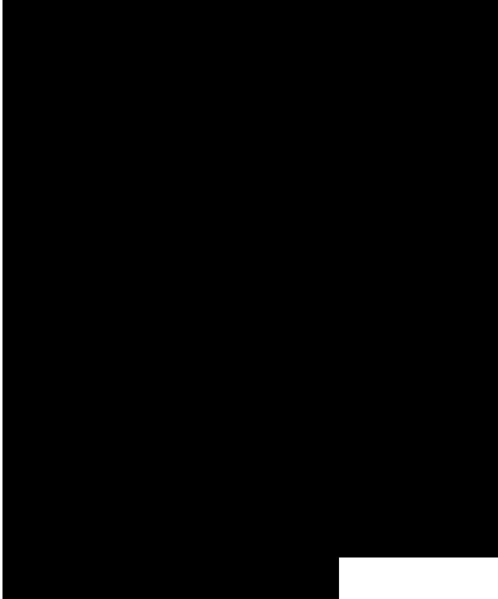
RFLP and rarefaction analyses of 16S crDNA libraries. A total of 185 Bacteria and 185 Archaea crDNA sequences from sediments with overlying orange-pigmented mats and 144 Bacteria and 148 Archaea clones from sediments with white-pigmented mats were grouped

according to RFLP patterns (data not shown).

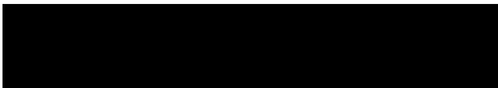
Rarefaction analysis (Fig. 1) and percent coverage were calculated to determine if a sufficient number of clones were examined to estimate diversity within each of the clone libraries sampled. Curves generated for crDNA clones obtained from both mat communities with the Bacteria primer sets did not indicate saturation (Fig. 1), while percent coverage was determined to be 92.4 and 89.6% for the orange- and white-pigmented mat libraries, respectively (15).

Although additional sampling of clones would be necessary to reveal the full extent of diversity, numerically dominant RFLP groups were obtained (Table 1). Specifically, one dominant bacterial phylotype from the white-pigmented mat (clone GoM 4702B-09) and orange-pigmented mat (clone GoM 5268WB-5) libraries comprised 15 and 18% of all clones, respectively (Table 1). In contrast, for those libraries obtained from both microbial

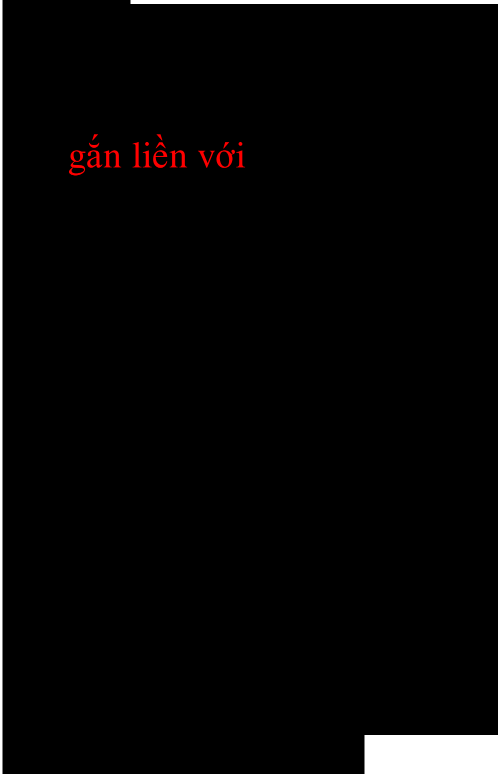
mat types with Archaea-specific primers, the curves indicated saturation and the percent coverage was 94.6 and 97.3% for the orange- and white-pigmented mats, respectively. Thus, a sufficient number of clones were sampled to be representative of the archaeal diversity in these libraries (Fig. 1). Numerically dominant phylotypes, containing 16 to 43% of all archaeal clones, were also obtained for each of these libraries (Table 2).



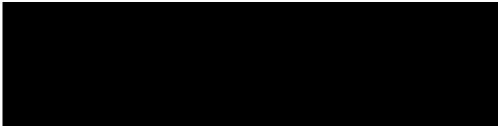
Phylogenetic diversity of metabolically active Bacteria.



Analysis of the 329 rRNA-derived Bacteria clones representing all three sediment depths associated with overlying orange- and white-pigmented microbial mat samples indicated a greater diversity relative to the Archaea clone libraries (Fig. 1). Bacteria clones were most similar to as yet uncultured bacterial lineages (Table 1). A total of 49 distinct RFLP patterns (data not shown) representing seven phylogenetic lineages were detected (Table 1). A considerable majority of the clones (93%) were representative of the phylum Proteobacteria (Fig. 2).



h-Proteobacteria. A total of 72% of all bacterial clones examined were most closely related to the



class Ò-Proteobacteria. Included was the most numerically dominant phylotype in the bacterial clone library, designated delta-3 (15% of the total library; Table 1). The delta-3 phylotype, most similar (99%) to a noncultured microorganism initially identified from the Cascadia Margin, was detected more frequently at 6 to 8 cm and 10 to 12 cm regardless of mat type (Fig. 3 and 4). In contrast to delta-3, the phylotypes delta-9 and delta-5 (Table 1) occurred three- to fivefold more frequently in sediments covered with orange-pigmented mats relative to clones from white-pigmented mats (Fig. 3). Phylotype delta-4 was found exclusively associated with the orange-pigmented mat (Fig. 3) and only at the upper (0 to 2 cm) depth (Fig. 4). Whereas the highest incidence of metabolically active delta-4, delta-5, and delta-9 phylotypes occurred in sediments associated with the orange- pigmented mats, phylotypes delta-1, delta-6, and delta-12 were most dominant in sediments associated with the white-pigmented mats (Fig. 3). These phylotypes exhibited a 3.8- to 11-fold-greater incidence in sediments associated with white-	chiếm ưu thế về mặt số lượng ở độ sâu các chuyên biệt Người ta thấy rằng gắn với chỉ	

pigmented mats relative to clones associated with overlying orange mats. While delta-1 was exclusively detected at 0 to 2 cm (Fig. 4), phylotypes delta-6 and delta-12 were most frequently detected (5- and 18-fold, respectively) at the lower depths (6 to 8 and 10 to 12 cm; Fig. 4).	được tìm thấy thường xuyên nhất tương ứng là 18 lần	
y-Proteobacteria. The second most dominant group of bacterial phylotypes was most similar to several noncultured microorganisms, including Beggiatoa sp. 'Monterey Canyon' (2) (Fig. 2), all clustering within the class y-Proteobacteria (22% of all clones; Table 1). The phylotypes associated with the overlying orange-pigmented mat most closely related to the Beg- giatoa sp. 'Monterey Canyon' were either predominantly (i.e., gamma-1) or exclusively (i.e., gamma-3 and gamma-4) located at 0 to 2 cm (Fig. 3 and 4). NGỌC QUÍ KẾT THÚC In contrast, phylotypes gamma-2 and gamma-5 (Fig. 3) were more frequently detected in sedi-ments covered with white-pigmented mats and were most similar to clones previously characterized as mussel endo-symbionts (Table 1). These were the only metabolically active y-Proteobacteria-related clones detected at 10 to 12 cm (Fig. 4).	gắn với chiếm ưu thế chuyên biệt	

Phylotypes gamma-2 and gamma-3 were most related (98% similar) to each other (Fig. 2). However, BLAST results indicated these phylotypes were 93% similar to two different environmental clones (Table 1).

e-Proteobacteria. The remaining Proteobacteria-related clones were located on two different clades within the class £-Proteobacteria (Fig. 2). The epsilon-1 phylotype, highly similar (97%) to a previously identified cold-seep clone (Table 1), occurred in sediments covered with orange- and white-pigmented mats and was not detected any more frequently at any particular depth (Fig. 3 and 4). In contrast, the epsilon-2 phylotype, 98% similar to an uncultured clone first isolated from the Japan Trench which had also been obtained by reverse transcription-pCR (21) (Table 1), was exclusively obtained from sediments associated with the overlying orange-pigmented mat (Fig. 3) and predominately from 0 to 2 cm (four of six clones; Fig. 4).

Nonproteobacterial lineages. In contrast to numerous Proteobacteria -related phylotypes, clones exhibiting similarity to

the classes Planctomycetacia, Verrucomicrobia, and Chloroflexi appeared to exhibit potential mat specificity. For example, Planctomycetacia-related clones, represented by four distinct phylotypes (n = 9; Table 1), were detected almost exclusively at lower sediment depths covered with white-pigmented mats (Fig. 3 and 4). The Verrucomicrobia- (n = 3) and Chloroflexi- related (n = 5) clones were detected at various sediment depths and associated exclusively with the overlying orange- pigmented mat (Fig. 3 and 4). Clones from each of these three classes were most closely related to environmental clones (Table 1) that have only been previously obtained from DNA-derived clone libraries. The remaining non-proteobacteria-related clones were most similar to the class Sphingobacteria and were detected in sediments covered with orange- and white-pigmented mats at 0 to 2 and 6 to 8 cm, respectively (Fig. 3 and 4)

Phylogenetic diversity of metabolically active Archaea. A total of 333 rRNA-derived Archaea clones, obtained from sediments with overlying orange- and white-pigmented microbial mats, grouped into 21 distinct RFLp patterns (data not

shown), and representative clones from all patterns were sequenced (Table 2). Interestingly, these 21 RFLp groups represented only two phylogenetic lineages, Crenarcheota and the Euryarchaeotal ANME-2 cluster of the order Methanosarcinales.

ANME-2. The majority of Archaea clones (95%; Table 2) were related to a distinct clade of Methanosarcinales known as ANME-2 (35). Members of this cluster have been detected previously in methane seep environments with sediment profiles indicative of anaerobic methane oxidation activity (19, 24, 35, 51). The ANME-2 cluster has been divided into four distinct subgroups, designated A, B, C, and D (Fig. 5) (31, 35). Clones representing all four of these subgroups were detected in this study (Fig. 5). Subgroup A was numerically dominant in the clone library (n = 145), while subgroup C exhibited the greatest intraclade genetic diversity (n = 7) relative to the other ANME-2 subgroups (Table 2). The phylotype ANME- 2A-1 comprised 43% of the total Archaea library (Table 2) and was most frequently detected in sediments covered with white-pigmented mats. We also observed a significantly greater ($P < 0.05$) number of ANME-2A-

1 clones at 0 to 2 cm (see Fig. 7).

In contrast, a comparable number of ANME-2B-1 clones, related to ANME-2 subgroup B, were detected in sediments

Fig. 4. Comparison between 16S crDNA Bacteria clones obtained from specific depths in sediments associated with orange- and white- pigmented mats. Clones are phylogenetically grouped according to sequence analysis data. Numbers along the abscissa denote unique phylotypes as determined by RFLP analysis and are consistent with the phylotype names in Table 1.

covered with both mat types (Fig. 6). This phylotype was recovered from sediments associated with orange-pigmented mats and only detected at 0 to 2 cm, while clones obtained from white-mat-covered sediments predominated at 10 to 12 cm (13 of 18 clones; Fig. 7).

Therefore, this metabolically active phylotype appeared to exhibit depth specificity relative to mat type. Clones related to the subgroup ANME-2C were dominated by two phylotypes, ANME-2C-1 and ANME-2C-2 (Table 2), and were most related to environmental clones previously isolated from a cold

methane seep locale in the Eel River Basin (35). Both of these phylotypes were more frequently detected in sediments associated with overlying orange-pigmented mats (Fig. 6) and were metabolically active at all sampled sediment depths (Fig. 7). However, ANME-2C-1 was significantly ($P < 0.05$) more active at the lower depths sampled (6 to 8 and 10 to 12 cm; Fig. 7).

The remaining five ANME-2C-related phylotypes were detected at a low frequency (10 of 333) and were predominately metabolically active at 0 to 2 and 6 to 8 cm (Fig. 7). The fourth ANME-2 subgroup, designated ANME-2D, was first observed in Archaea-specific 16S rRNA gene libraries derived from total DNA extracted from sediments directly associated with surface-breaching gas hydrate (31). In the present study, two distinct phylotypes from this subgroup were identified (Fig. 5). The phylotype denoted ANME-2d-1 was closely related (99.8% similar) to the dominant ANME-2D phylotype reported by Mills et al. (31) and was most frequently (fivefold) detected in orange-mat-covered sediments (Fig. 6). However, the ANME-2D-2 phylotype was genetically divergent, having only 92% sequence similarity to GoM

GC234 606R (31) (Fig. 5) and was not specific to any particular mat type.

Crenarchaeota. The remaining 5% of the archaeal clones (n = 15) were grouped into six distinct RFLP patterns (data not shown) forming three clades within the Crenarchaeota lineage (Fig. 5). Clones representing these six phylotypes were most similar to sequences obtained from noncultured microorganisms (Table 2 and Fig. 5). Phylotype Cren-1 represented a majority of the Crenarchaeota-related clones (9 of 15) and was most related (99% similar) to a 16S rRNA gene sequence isolated from surface sediments in the North Sea (55) (Fig. 5). In addition, phylotype Cren-1 was predominately active only at 0 to 2 cm (eight of nine) and exclusively associated with the overlying orange-pigmented mat. Interestingly, with the exception of the single clone associated with the Cren-2 phylotype, all other Crenarchaeota-related clones obtained in the present study were isolated from orange-mat-covered sediments (Fig. 6).

DISCUSSION

This study is the first to report the composition of the metabolically active members of the archaeal and bacterial com-

munities in gas hydrate sedimentary systems in the Gulf of

fig. 6. Comparison between 16S crDNA Archaea clones obtained from sediments associated with overlying orange- and white-pigmented mats. Clones are phylogenetically grouped according to sequence analysis data. Numbers along the abscissa denote unique phylotypes as determined by RFLP analysis and are consistent with the phylotype names in Table 2.

Mexico. Moreover, to the best of our knowledge, this is also one of the first characterizations of metabolically active Ar- chaea from marine environments as determined by rRNA analysis and one of the first microbial community depth profiles of sediments associated with overlying microbial mats in cold-seep habitats. Inagaki et al. (21) recently reported that e-

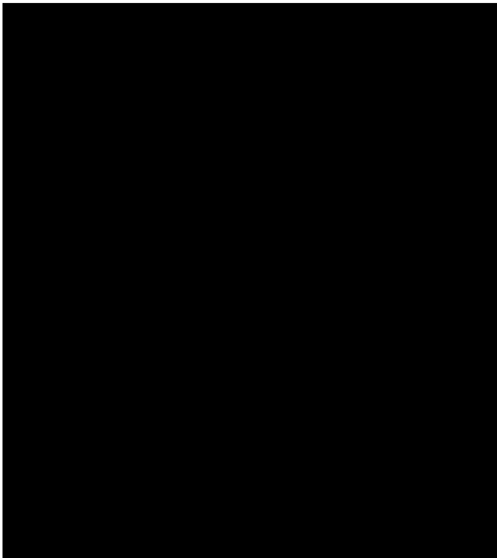
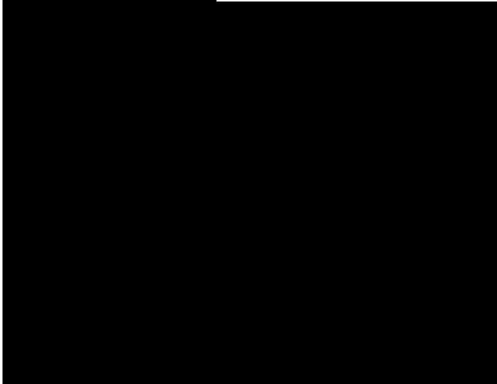


fig. 7. Comparison between 16S crDNA Archaea clones obtained from specific depths in sediments associated with overlying orange- and white-pigmented mats. Clones are phylogenetically grouped according to sequence analysis data. Numbers along the abscissa



denote unique phylotypes as determined by RFLP analysis and are consistent with the phylotype names in Table 2.
lacking microbial mats, £-Proteobacteria dominated the bacterial clone libraries (31). However, in the present study, fewer than 4% of the metabolically active phylotypes detected at 0 to 2 cm belonged to the £-Proteobacteria. Instead, the clone library of Bacteria at 0 to 2 cm was dominated by y-Proteobac- teria and h-Proteobacteria (45 and 48%, respectively), while the libraries of Bacteria at 6 to 8 cm and 10 to 12 cm were dominated by h-Proteobacteria.

All y-proteobacteria-related clones derived from sediments covered with either orange- or white-pigmented microbial mats were most similar to either Beggiatoa spp. or macrofaunal endosymbionts (93 to 99% similar). Surprisingly, all of the Beggiatoa-related clones were most similar to Beggiatoa sp. 'Monterey Canyon' (2), providing a possible biogeographical link between these two distinct cold-seep environments. As geologic evidence has shown that the presence of a deep water current flowing between the Gulf of Mexico and the Eastern pacific

was disrupted 4.6 million years ago as a result of the rise of the Isthmus of Panama (17), it is tempting to speculate that these Beggiatoa populations originated from a common ances- tor(s) separated by this event. The occurrence of other y-pro- teobacterial phylotypes related to previously identified endo- symbiont clone sequences may be explained by the presence of numerous juvenile clams and shrimp observed during microscopic examination of intact, unprocessed sediments (0 to 2 cm). Whether these endosymbionts are free living in the sediment or were detected as a result of disruption or breakage of the juvenile clams and shrimp cannot be determined in this study.

While the vast majority of the metabolically active y-Pro- teobacteria phylotypes detected in this study appeared to be constrained to 0 to 2 cm, four distinct clades of active h-Pro- teobacteria remained numerically dominant at all three depths. As many members of the h-Proteobacteria are known sulfate- reducing bacteria, these clades are likely to be important players in sulfur cycling. Although not determined in the present study, previously measured porewater sulfate concentrations (>25 mM) from

Gulf of Mexico site GC234 sediments associated with overlying microbial mats did not exceed the sulfate concentrations in sediments lacking mats (22). The rates of sulfate reduction in sediments covered with mats, however, were several orders of magnitude greater along a 0- to 12-cm depth profile relative to comparable sediments lacking microbial mats (22). A corresponding increase in H2S concentration was detected as sulfate concentrations decreased with increasing sediment depth (22). Such concentrations and rates are similar to previously characterized Beggiatoa sp. mat-associated sediment porewater from other cold-seep environments (3, 35) as well as a Gulf of Mexico study conducted at GC185 (1).

We hypothesize that the predominance of active h-Proteo- bacteria detected at the lower depths may be explained by two different mechanisms. First, upward flow of subsurface fluids channeled around microbial mats may result in a downward fluid flux through the mat (57). Sulfate-rich seawater is pumped deeper into the mat-covered sediments than surrounding sediments lacking mats. Thus, the microbial mats would provide a localized increased concentration of

sulfate at lower depths, facilitating overall higher rates of sulfate reduction. Second, anaerobic sulfur oxidation due to Beggiatoa sp. activity would replenish sulfate throughout the sediment profile. Although there was a sevenfold increase in the total number of meta- bolically active Beggiatoa sp. clones detected at 0 to 2 cm, Beggiatoa-related clones were also detected at 6 to 8 cm and 10 to 12 cm. Such results were perhaps not surprising, as previous reports have demonstrated the ability of Beggiatoa spp. to migrate below 10 cm to reach elevated concentrations of H2S (2, 33).

Planctomycetales-related clones were more frequently detected at the lower depths (6 to 8 cm and 10 to 12 cm) in sediments covered with orange- and white-pigmented microbial mat communities. previous studies have demonstrated the breadth of physiological characteristics of this phylum (14, 26, 44), including a possible link between some members of the Planctomycetales to the anaerobic oxidation of ammonia (45, 46). This process, known as ANAMOX and described by a metabolic pathway first reported by Van de Graaf et al. (54), requires ammonia and nitrite in an anaerobic environment to produce dinitrogen gas (47, 48).

Ammonium concentrations in previously characterized porewater from one of our study sites (GC234) were fourfold higher in Beggiatoa sp. mat-covered sediments at 10 to 12 cm (>30 ^M) relative to sediments lacking microbial mats (5 ^M) (22). Although nitrite concentrations were not determined, nitrate concentrations in pore- water from GC234 sediments with microbial mats were highest at the surface (>20 ^M) and decreased to less than 2 below 2 cm (22).

The nitrite source required for ANAMOX may be derived either from the incomplete reduction of nitrate or from the advective flow of nitrite-bearing seawater through the Beggia-toa-covered sediment, as has been reported by Weber and Jorgensen (57). Therefore, an increased concentration of nitrite and ammonia may be attributed to the presence of Beg- giatoa sp. mats. Thus, we theorize that the Planctomycetales- related clones detected in this study are dependant on the presence of the Beggiatoa sp. mat community.

Methane concentrations and anaerobic oxidation of methane rates previously determined for GC234 sediments covered with Beggiatoa sp. mats have been shown to be several orders of

magnitude higher than that of control sediments lacking microbial mats (22). In the present study, the vast majority (95%) of Archaea clones obtained from the three sediment depths also sampled from Gulf of Mexico sites GC234 and GC185 were phylogenetically related to the ANME-2 group of the order Methanosarcinales, proposed candidates for anaerobic oxidation of methane.

ANME-2-related sequences have been isolated from total DNA extracted from other cold-seep environments (19, 24, 31, 35) but have never represented such a majority of the sequences as obtained in this study. This study also represents the first archaeal clone library containing sequences related to all four ANME-2 subdivisions (A, B, C, and D) (31, 35). Interestingly, the ANME-2C-related clones do not form a Gulf of Mexico-specific cluster, as was observed in other phylogenetic analyses of Gulf of Mexico hydrate-associated sediments (24, 31). However, one of the two ANME-2D-related phylo- types was only 92% similar to previously identified ANME-2D sequences from the Gulf of Mexico (31) and thus may represent a novel lineage within the ANME-2D clade.

The uniqueness of the Archaea clone libraries constructed in this study may be a result of the environmental conditions associated with an overlying microbial mat community or of this study's being the first to characterize metabolically active archaeal communities from a cold-seep locale. PCR primer bias seems less likely, as the primers employed in this study have been used in another Gulf of Mexico study that resulted in more diverse libraries that included sequences related to Methanomicrobiales, Archaeoglobus, non-ANME-2 Meth- anosarcinales, and Crenarchaeota (H. J. Mills, R. M. Martinez, S. Story, and p. Sobecky, unpublished data).

Based on our current findings characterizing the metaboli- cally active fraction of the bacterial and archaeal communities in conjunction with recent geochemical data and microbial rate measurements from Beggiatoa-covered sediments (22), we propose the following. Beggiatoa spp. serve as keystone members of the seep community owing to their ability to (directly and indirectly) influence the metabolic activity of h-Proteobacteria, Planctomycetales, and ANME archaea. The end products of

Beggiatoa--mediated anaerobic sulfur oxidation (sulfate and ammonia) and an increase in advective flow rate into the mat (57) would result in higher concentrations of reactants available for h-Proteobacteria and Planctomycetales. Recent findings by Joye et al. (22) lend support to this hypothesis, as they detected an increase in sulfate and ammonium concentrations and microbial sulfate reduction rates in Gulf of Mexico GC234 sediments with overlying microbial mats. In addition, the increased rate of sulfate reduction and advective flow of organic material into the sediment can promote a more conducive environment for anaerobic oxidation of methane (reviewed in reference 53). The predominance of ANME-related clones (regardless of sediment depth) and reported high rates of anaerobic oxidation of methane (22) support this general hypothesis.		
In conclusion, this study presents some of the first molecular phylogenetic data describing the fraction of the metabolically active Bacteria and Archaea communities in Gulf of Mexico cold-seep habitats. Such information provides insights into the interconnection and interdependency of the microbial populations residing in	Tóm lại, đây là nghiên cứu đầu tiên trình bày một số dữ liệu phát sinh loài ở cấp độ phân tử mô tả một phần cộng đồng vi khuẩn và cổ khuẩn có hoạt động trao đổi chất ở Vịnh Mexico trong môi trường sống phun lạnh. Các nghiên cứu này giúp chúng ta hiểu sâu hơn về mối liên hệ và sự phụ thuộc lẫn nhau của các quần thể vi sinh vật sinh sống	

sediments associated with overlying mat communities dominated mainly by Beggiatoa spp. …………………………………… …………………………………… …………………………….	trong các trầm tích gắn liền với các cộng đồng thảm bao phủ, trong đó Beggiatoa spp là loài vi khuẩn chiếm ưu thế. …………………………………… …………………………………… …………………………….	